Chapter 6

Origin, Recognition, Signaling and Repair of DNA Double-Strand Breaks in Mammalian Cells

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Abstract

A chromosomal double-strand break (DSB) can arise from multiple sources including ionizing radiation and DNA replication itself. An understanding of the intricate protein pathways that recognize DSBs and recruit the DNA repair and cell cycle checkpoint machinery is developing rapidly. The ATM kinase plays an early, pivotal role in the signaling process by detecting DSBs and relaying this information to numerous downstream transducer and effector proteins. Within minutes after DSBs occur, ATM undergoes inter-molecular autophosphorylation at Ser^{1981}, which converts it to an active monomer. $ATM^{Ser1981\text{-}P}$ immediately phosphorylates histone H2AX over a megabase region of DNA surrounding a DSB. Discrete nuclear foci of phosphorylated H2AX (γH2AX) are visible by immunofluorescence and appear to be true markers of DSBs. MDC1 and 53BP1, transducer proteins that contain two C-terminal BRCT domains, are also phosphorylated by ATM and colocalize faithfully with γH2AX. Subsequent transducers and effectors include the Mre11-Rad50-NBS1 complex (both transducer and effector), and the breast cancer susceptibility proteins BRCA1 (a transducer) and BRCA2 (an effector). BRCA2 interacts directly with DNA and the Rad51 strand-transferase to help initiate homologous recombination. When the DNA replication machinery is chemically inhibited or encounters a damaged template containing single-strand breaks or blocking lesions, replication forks may arrest, collapse into one-sided DSBs, and require recombinational repair to be reestablished. This recovery process is dependent on the ATR kinase acting in concert with the Rad17-Rfc clamp-loader complex and the Rad9-Rad1-Hus1 clamp complex. Modifiers of DNA topology, such as BLM and WRN helicases associated with Bloom and Werner syndromes, assist in preserving chromosomal continuity during replication. These proteins are thought to resolve anomalous replication intermediates that arise at stalled forks, thereby preventing aberrant recombination for unrepaired DSBs. Overall, the precise nature of a DSB likely determines whether ATM or ATR is utilized to initiate the damage-response pathways.

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Replication-Independent Double-Strand Breaks (DSBs)

Origins of DSBs

Double-strand breaks (DSBs) are of fundamental importance in many fields of biology. The incorrect repair of DSBs often results in chromosomal rearrangements, which are considered to be a major initiating factor in carcinogenesis. Cancer cells generally exhibit numerous structural rearrangements (i.e., deletions, exchanges, duplications, and inversions) as well as increased numbers of chromosomes. Progression of malignancy often correlates with increased chromosomal instability and plasticity, which are driven by escalating defects in DNA repair processes[1] and cell cycle checkpoint functions.[2-4] The cellular lethality of ionizing radiation (IR) occurs largely through the production of DSBs. Many cancer treatments rely on the ability of IR and chemical agents (e.g., bleomycin) to produce DSBs that can be targeted to preferentially eradicate tumor cells versus damaging normal tissues. Thus, understanding the quantitative yields of DSBs and the molecular mechanisms that eliminate them is a central issue in cancer biology and radiation biology.

The yield of breaks produced by IR is estimated to be ~35 DSBs per diploid G1 cell per Gy (measured at doses ≥ 20 Gy), compared with a value of ~1000 single-strand breaks (SSBs) per Gy.[5-8] Recent estimates of DSB yield measured by the frequency of IR-induced γH2AX foci[9,10] at doses between 0.001 and 3 Gy give a value that is very similar to the ~35 breaks per Gy determined at high doses by pulsed-field gel electrophoresis.[11] However, estimates based on premature chromosome condensation (PCC), which allows visualization of chromosomes in G_1 nuclei, are considerably lower at 5 to 6 DSB per Gy per cell.[12,13] The reason(s) for this discrepancy is unclear. One possible explanation is that IR-generated DNA fragments arising from two or more DSBs in relatively close proximity would not be microscopically distinguishable from a single DSB. Second, in the PCC method some fraction of the rapidly repaired DSBs will be missed because of the 15-20 min post-IR incubation at 37°C required to produce cell fusion and chromosome condensation. This fraction could be as high as 65%.[14] IR also produces oxidative base damage, but the amounts of damaged bases[8] per Gy of radiation are estimated to be 10-100 fold lower than the steady state levels (~1.5×10^5 oxidative base lesions per human cell[15]) produced by normal oxygen metabolism.[16]

Recently though, it has become apparent that IR produces clustered oxidative base damages and SSBs on opposite strands of the DNA molecule, which can develop into DSBs and represent potentially lethal lesions.[17-19] Clusters of closely opposed SSBs, oxidized purines, oxidized pyrimidines, or oxidized abasic sites within a few helical turns are estimated to comprise at least 70% of the complex lesions produced in cells.[19] These clustered lesions are likely difficult to repair[20] and may get converted to DSBs through processing by base-excision repair enzymes, or by interaction with DNA replication forks as they encounter clustered lesions.

DNA Repair Systems that Act on DSBs

Cells possess complex, highly efficient mechanisms for detecting DSBs and signaling their presence to the DNA repair and replication machinery. In this review, we address what is known about these recognition and information transfer systems outlined in (Fig. 1). Our understanding of how cells respond to DSBs has developed rapidly with respect to the enzymatic machinery that performs repair. Considerably less is known about the preceding events of detecting/sensing/recognizing breaks and the signaling processes that recruit DNA repair systems to the sites of damage. The two major pathways that repair DSBs are referred to as nonhomologous end joining (NHEJ)[21,22] and homologous recombinational repair (HRR).[23-25] In this review, we address what is known about these recognition and information transfer systems, along with a brief, updated summary of HRR (Fig. 5).

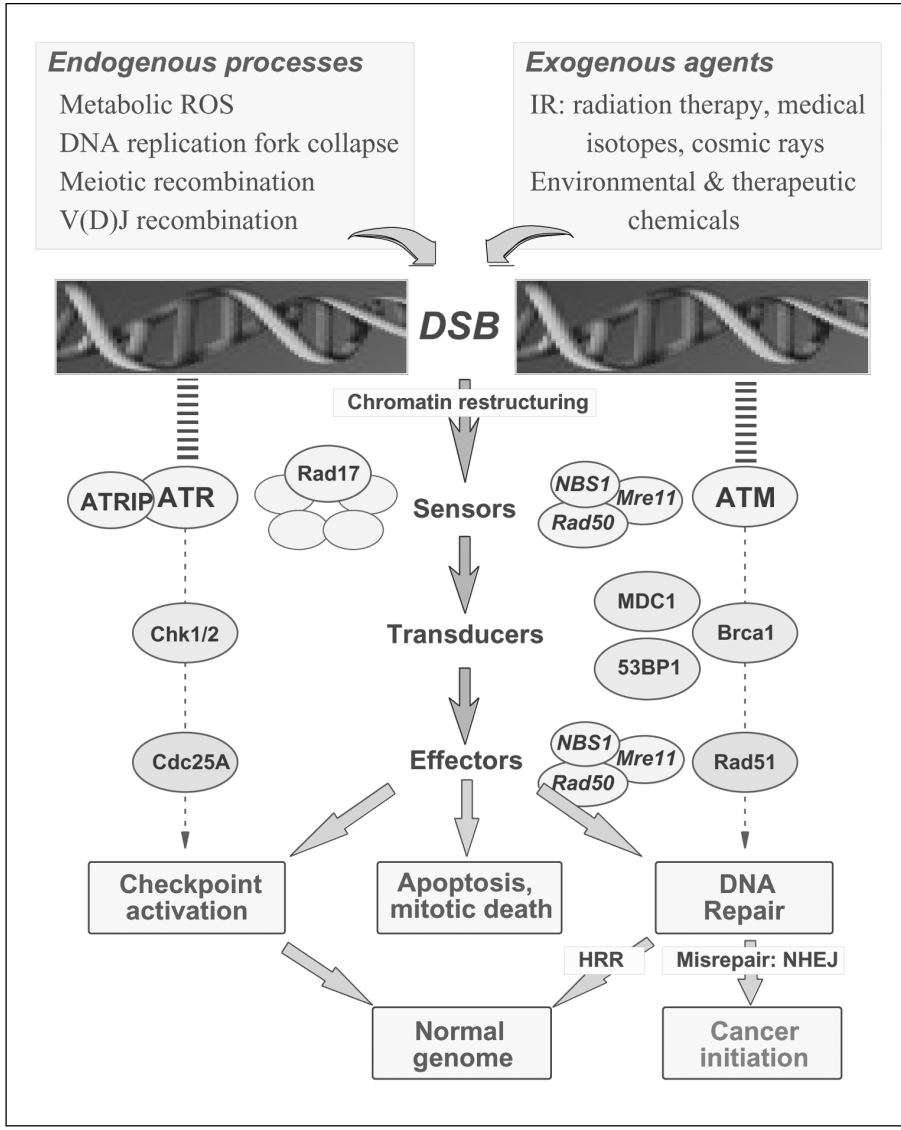

Figure 1. Recognition and signaling of DSBs. DSBs can arise directly through the action of radiation or chemicals, or indirectly though the enzymatic action of DNA repair enzymes on clustered oxidative lesions or the interaction of DNA replication forks with single-strand breaks. DSB detection is followed by signaling reactions (often phosphorylations) that implement repair and checkpoint functions, transcriptional changes, or possibly apoptosis if a cell is severely damaged. Examples of transducers are given.

DSBs occur normally during meiosis to initiate strand exchange between homologous chromosomes and in hematopoietic cells during gene processing through V(D)J recombination, which mediates antibody diversity and gene rearrangements for T cell receptors. These highly regulated, programmed DSB-mediated processes are normally extremely accurate and utilize many of the same DNA break-processing enzymes that repair spontaneous or

agent-induced DSBs. High levels of spontaneous DSBs and chromosomal rearrangements are observed in mouse cells carrying null mutations in the genes of the NHEJ complexes composed of Ku70-Ku86-DNA-PKcs or LIG4-XRCC4.[26-28] The levels of chromosomal breaks can be reduced by lowering the oxygen tension from 20% to 3%.[29] Elevating the level of reactive oxidative species, rather surprisingly by overexpressing a transgene for the antioxidant enzyme superoxide dismutase 1 (SOD1), increases chromosome breakage. In SOD1-overexpressing cells, reducing oxygen to 3% also reduced chromosomal aberrations. The observation that oxidative damage results in spontaneous chromosome breaks may explain the neuronal degeneration and premature aging that typify mice having NHEJ mutations.[30-34]

However, not all NHEJ-defective cell lines (i.e., Ku70 and Ku80 mutants in hamster CHO and chicken DT40 backgrounds) display markedly increased levels of spontaneous chromosomal aberrations.[35-37] The reason for the significant differences among cell types is not clear, but it is noteworthy that both the CHO and DT40 lines are defective for Tp53. Perhaps this defect allows for increased DNA-PKcs-independent end joining in the absence of the DNA-PK or LIG4-XRCC4 complexes.

Central Role of the ATM Kinase in DSB Signaling

The large ATM (ataxia telangiectasia mutated) and ATR (AT and Rad3-related) kinases have come into focus as early, central participants in the DNA damage recognition and signaling processes (Fig. 2 and Fig. 6).[3,38-41]These functionally related proteins phosphorylate a multitude of substrates and appear to exist in vivo in high molecular weight complexes of >2 x 10^6 Da,[42,43] which may contain many other damage-response proteins.[44] Figure 2 expands the theme of (Fig. 1) by depicting numerous phosphorylation events as well as functionally important protein interactions. Exposure of cells to IR immediately activates the ATM kinase (3056 a.a.; $Tel1^{Sp}$ and $Tel1^{Sc}$ homologs in yeasts),[45,46] and ATP can also induce activation of by a mechanism involving autophosphorylation.[47] A major advance came with the discovery that IR-induced activation occurs through intermolecular autophosphorylation of Ser^{1981}, which causes dissociation of ATM dimers and enhancement of kinase activity.[48] After IR, phosphorylation of Ser^{1981} is maximal within 5 min and saturates at a dose of ~40 cGy.[48] Upon activation, ATM phosphorylates histone H2AX (modification referred to as γH2AX);[49] DNA-PK and ATR also contribute to this modification.[50-53] Although ATM binds preferentially to DNA ends in vitro,[54] the in vivo activation likely results from changes in chromatin structure instead of DNA binding.[48] ATM phosphorylates numerous key proteins that often appear in nuclear foci (described below) and that mediate checkpoints and DNA repair: namely 53BP1, MDC1/NFBD1, Chk1, Chk2, NBS1, BRCA1, and FANCD2. Altogether, ATM has more than 20 substrates, as recently reviewed in more detail.[25] Thus, throughout the cell cycle ATM acts as a master regulator and coordinator in the initial response to DSBs that are not associated with replication forks. ATM is also activated by agents such as methylating chemicals that do not directly cause DSBs, but lead to lesions that are subsequently converted to DSBs.[55] The closely related ATR kinase discussed below may serve as a partial backup system for ATM and help to reinforce at later times the phosphorylating signaling initiated by ATM (see discussion in ref. 41).

The fact that ATM is responsible for phosphorylating proteins that implement repair and checkpoint functions suggests that ATM itself might concentrate at sites of DSBs. Indeed, within 5 min after IR, the Ser^{1981}-phosphorylated form of ATM begins to form foci that colocalize with γH2AX foci, and these become distinct foci by 60 min.[48] Under conditions of detergent extraction to remove nucleoplasmic proteins, a portion of the total ATM pool becomes resistant to extraction and is detected in nuclear aggregates immediately after DSB formation.[56] These aggregates are much more diffuse than the distinct foci formed by γH2AX.

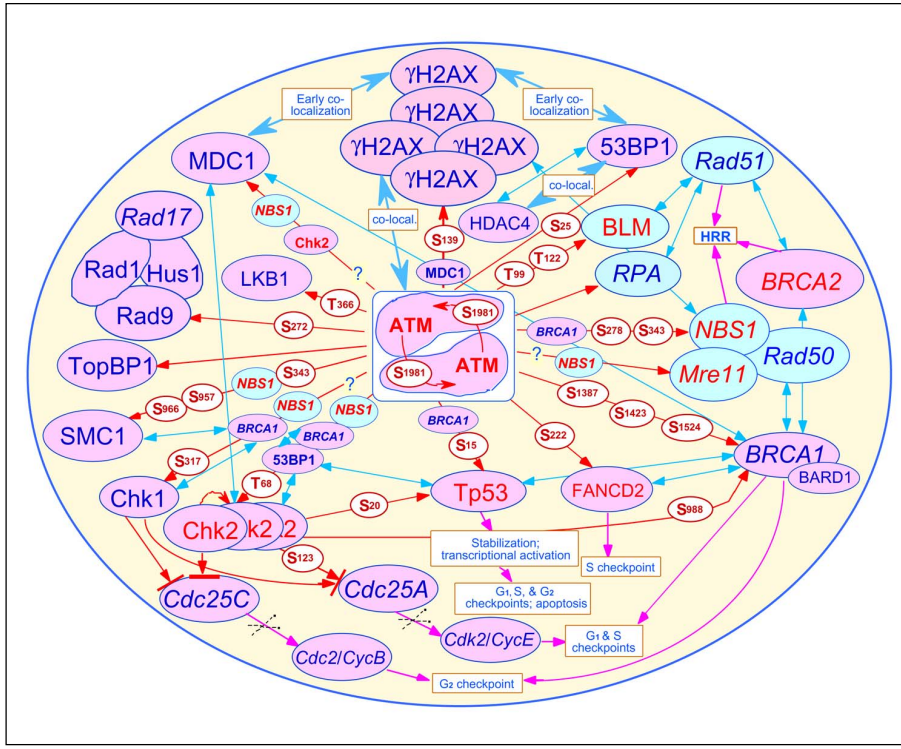

Figure 2. ATM mediated phosphorylation events and interactions that implement signaling, repair, and checkpoint functions in response to replication-independent DSBs. Within 60 sec after irradiation, phosphorylation of histone H2AX (referred to as γH2AX) occurs in discrete foci at sites of DSBs. These foci, which contain ~1000 or more γH2AX molecules in a 10^6 bp-region of DNA, likely recruit the numerous proteins that signal the presence of damage, conduct DNA repair, initiate checkpoints to halt cell cycle progression, or facilitate apoptosis in heavily damaged cells. These initial chromatin modifications must create high-affinity sites that localize such damage-response proteins. The choice of repair pathway, NHEJ or HRR, is probably dictated by the position of the cell in the cell cycle and by the structure of the DSB (e.g., one-sided versus two-sided breaks; see (Fig. 7C). A DSB occurring in a region of DNA that has replicated may be specifically channeled through HRR so that mutations can be avoided. This pathway specificity may be conferred by the proteins that are present in the vicinity of the break, as well as an altered state of the replicated chromatin, which distinguishes it from unreplicated chromatin in G1 cells and unreplicated DNA regions in S cells. Proteins with names in red are involved in human genetic disorders and proteins in italics are required for viability of dividing cells. Specific phosphorylation steps that are thought to be functionally important have been reported: H2AX,[49] BLM,[312] NBS1,[61-64] BRCA1,[222,313-317] FANCD2,[167] Tp53,[45,46,318] Chk1,[105,107] Chk2,[319-324] SMC1,[166,325] and Rad9.[326] Additional ATM-dependent phosphorylation targets are RPA,[327-329] 53BP1,[91,93] TopBP1,[127] LKB1,[330] MDC1/NFBD1,[58,79-81] and possibly Mre11.[65,331] Chk2 also phosphorylates BRCA1.[332] BRCA1 is required for phosphorylation of 53BP1, Tp53, and NBS1, [356] and NBS1 is required for the phosphorylation of MDC1, SMC1, Chk1, 53BP1, and Mre11. Other phosphorylation requirements are also indicated by proteins on the red lines. Question marks indicate that the phosphorylation could be indirect. The heavy blue arrows show proteins that colocalize within minutes after IR damage. The interactions, determined by coimmunoprecipitation, marked by blue arrows have been reported for MDC1-Chk2,[80] MDC1-BRCA1,[116] BLM-Rad51,[333] RPA-Rad51,[122] BRCA2-Rad51,[334] BRCA1-BARD1,[335] BRCA1-BRCA2,[336] BRCA1-Tp53,[337] BRCA1-Rad50,[338] 53BP1-Tp53,[87] 53BP1-Chk2,[57] 53BP1-BRCA1,[57] 53BP1-γH2AX,[91] 53BP1-HDAC4,[97] and BRCA1-FANCD2.[339]

Although the sensor proteins that first recognize DSBs are not well understood, ATM is a major candidate sensor protein. It could act alone or in combination with other proteins discussed below such as 53BP1[57] and MDC1/NFBD1[58] that localize within minutes to sites of DSBs. It is noteworthy that certain ATM mutations display a dominant negative phenotype in the heterozygous state, both in humans and mice.[59,60] This situation could arise if mutant ATM binds and sequesters partner proteins into dysfunctional complexes that compete with normal complexes for DNA substrates. Although ATM is required to phosphorylate both NBS1 and Mre11,[61-65] genetic evidence suggests that the Mre11-Rad50-NBS1 (MRN) complex acts upstream of ATM, at least for some signaling events.[66] Mre11-defective human cells show reductions in detergent-resistant retention of ATM protein, ATM kinase activity, and phosphorylation of downstream targets.

Origins of Nuclear Foci that Form in Response to DSBs

When cells are exposed to DNA damaging agents, the redistribution and subnuclear localization of specific proteins can be monitored to infer which proteins are important in damage recognition, signaling, checkpoint implementation, and repair. In mammalian cells, Rad51 protein, involved in homologous recombination, was one of the first proteins detected in discrete nuclear foci using immunofluorescence on mitotic and meiotic cells[67,68] (Fig. 3A). The cytological visibility of these foci can be readily explained by the fact that Rad51 forms nucleoprotein filaments that can contain hundreds of Rad51 molecules. It is estimated that ~100 fluorophore molecules localized within a very small volume are necessary for a visible focus.[69] Many other proteins discussed below also form foci, but not all of these are expected to assemble en masse as multimeric functional complexes like Rad51. For example, the MRN complex is a key component in the processing of DSB termini,[70-72] and the MRN complex forms foci. Although the precise biochemical roles of this complex are not understood, only one or a few of these complexes, as a catalytic component, may be needed to produce single-stranded tails at the termini of DSBs prior to their repair by homologous recombination. Yet, focus formation may arise from the creation of multiple high-affinity binding sites for MRN in the vicinity of the DSB, thus causing numerous MRN complexes to concentrate at the modified site. This idea is illustrated by the observation that foci of Rad52 and Rad54 are highly dynamic structures.[73] These foci exhibit rapid exchange of these proteins, which are recruited independently with differing mobility.

γH2AX Formation As a Marker of Radiation-Induced DSBs: Impact on Checkpoints and Repair

Recent developments suggest the possibility that units of higher order chromatin structure may facilitate the detection of DSBs within DNA. In response to the introduction of DSBs, the minor histone H2AX is rapidly phosphorylated in a dose-dependent manner on Ser^{139} (located four amino acids from the carboxyl terminus), yielding the form designated γH2AX.[49] Each DSB produces ~2000 γH2AX molecules and results in the modification of H2AX over a region corresponding to ~ 2 Mbp,[49] which is the equivalent of 0.03% of the chromatin. An antibody specific for the modified C-terminus of H2AX reveals that γH2AX appears as discrete nuclear foci within 1 min after exposure of cells to ionizing radiation.[50] Cells in all phases of the cycle, including mitosis, show foci.[50,53]. Importantly, the number of foci agrees with the estimated number of induced DNA DSBs.[10,11,50] Significant phosphorylation of H2AX was also observed after treatment with the DSB-inducing agents neocarzinostatin, bleomycin, and etoposide, whereas UV irradiation and the DNA methylating agent methyl methanesulfonate did not produce γH2AX.[51] This pattern further supports the idea that γH2AX phosphorylation occurs specifically in response to DSBs. Recent studies show that the formation of γH2AX is severely reduced in DNA-damaged ataxia telangiectasia cells and that the

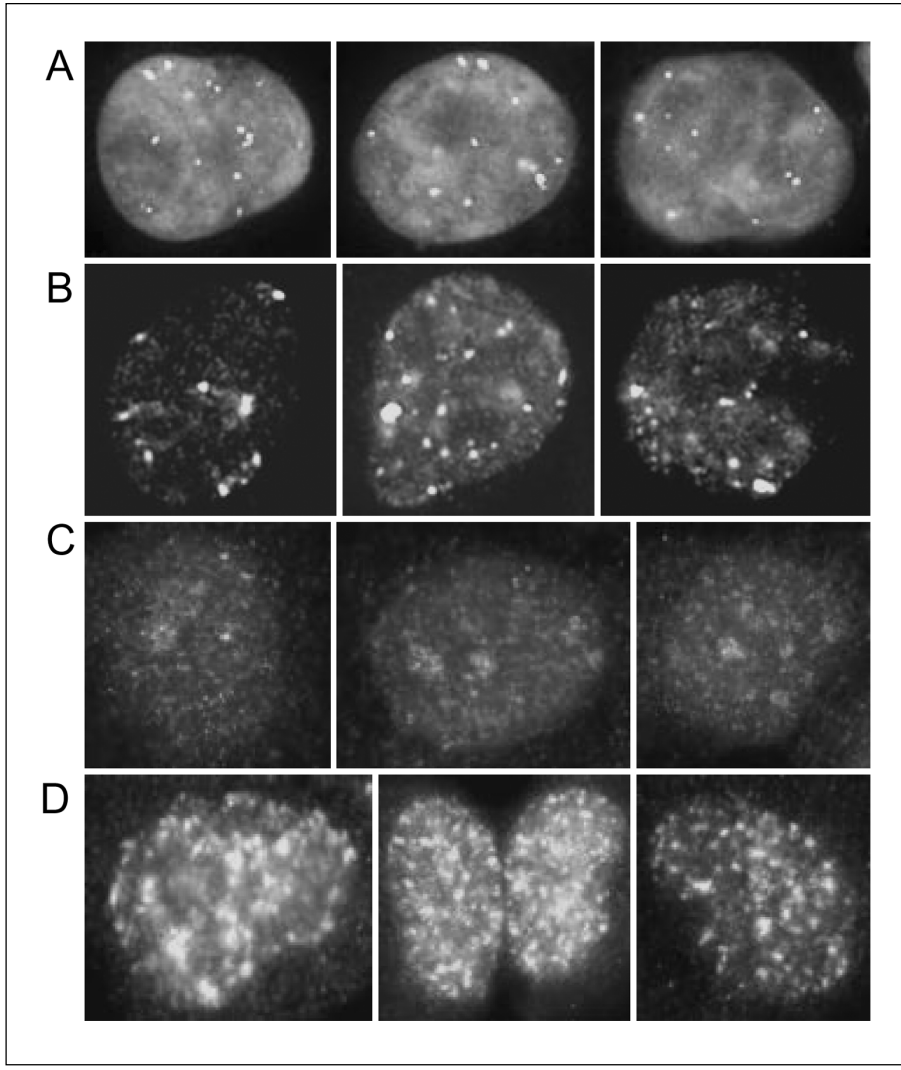

Figure 3. Rad51 and γH2AX nuclear foci produced by ionizing radiation. (A) CHO AA8 cells irradiated with 10 Gy and stained with Rad51 antibody and DAPI after 4 h. (B) Unirradiated CHO AA8 cells stained with γH2AX antibody. Putative S phase cells are shown; many cells had no foci. A recent study suggests that only the most intense foci represent true DSBs,[134] which emphasizes the importance of calibrating the scoring of foci. (C) Unirradiated GM637 fibroblasts stained with γH2AX antibody. (D) GM637 cells treated with 6 Gy and stained with γH2AX antibody after 30 min.

residual level could be attributed to DNA-PK activity [51,74] NBS cells (Nijmegen Breakage Syndrome; NBS1 is part of the MRN complex) have normal γH2AX focus formation,[75] but depletion of the MDC1 signal transducer discussed below reduces focus formation.[58] IR-induced H2AX phosphorylation is a highly conserved process that is present in vertebrates, Drosophila, and yeast.[50] γH2AX is formed in response to DSBs arising by diverse means: directly from environmental insult by radiation or chemicals, collapse of DNA replication forks, and pro-

grammed processes that enzymatically introduce DSBs (e.g., meiosis). The trigger for γH2AX formation may involve topological changes in the DNA, such as the degree of super coiling.

The biological importance of γH2AX formation in maintaining chromosome stability is clearly revealed by the phenotypes of mice carrying knockout mutations in one or both copies the *H2AX* gene.[76,77] *H2AX*$^{\Delta/\Delta}$ mice are growth retarded, radiation sensitive, immune deficient, and defective in spermatogenesis. The IR sensitivity of *H2AX*$^{\Delta/\Delta}$ ES (embryonic stem) cells is increased ~3-fold,[77] whereas the sensitivity of immortalized MEF (mouse embryonic fibroblast) cultures is increased only ~1.6-fold, but nevertheless these latter cells were shown to have reduced DSB repair.[76] Spontaneous chromosomal aberrations are also markedly elevated, e.g., from 5% in heterozygous *H2AXFlox*$^{\Delta}$ controls to 22% in *H2AX*$^{\Delta/\Delta}$ ES cells.[77] Although checkpoint functions in all phases of the cell cycle were considered to be normal in *H2AX*$^{\Delta/\Delta}$ cells following 10 Gy irradiation,[76,77] at lower doses a clear G2 checkpoint defect was seen in both mouse B cells and MEFs.[78] Thus, H2AX phosphorylation signals for both checkpoint activation and repair. In summary, the pleiotropic phenotype of *H2AX*$^{\Delta/\Delta}$ mice is caused by defects in signaling that include impaired recruitment of MDC1, 53Bp1, NBS1, and Brca1 (but not Rad51) into IR-induced foci.[58,76,77]

Recruitment and Colocalization of Signaling and Repair Proteins to Sites of γH2AX Foci

In this section we outline the characteristics of many proteins that are implicated in signaling, checkpoints, and repair through their redistribution within the nucleus in response to DNA breakage. Some of these proteins, such as MDC1/NFBD1 and 53BP1, appear to arise as quickly as, and coincident with, γH2AX foci while other foci (e.g., Rad51) arise much later. By further example, colocalizing γH2AX-BRCA1 foci were reported to appear sooner than γH2AX-Rad50 foci,[75] implying that BRCA1 may act upstream of the Rad50 complex (MRN, discussed below). Figure 4 summarizes available information on the order of appearance of foci, including colocalizations. Certainly there are numerous pitfalls in deciphering the significance of focus formation. Foci studies performed at high doses (e.g., > 5 Gy) and many hours after exposure will be much more difficult to interpret than studies terminated minutes after irradiation at low doses (e.g., < 1 Gy). The remainder of this section summarizes the characteristics and significance of many proteins that have been shown to form nuclear foci.

MDC1/NFBD1

Very recently a new nuclear human protein, MDC1 (mediator of DNA checkpoint; also called NFBD1 for nuclear factor containing two BRCT domains at the C-terminus; 2089 a.a.), which constitutively binds to chromatin, was identified as a very early participant in the recognition and signaling process.[58,79-82] Because of its C-terminal BRCT domains, MDC1 is a candidate functional homolog of Rad9^{Sc}, one of the first checkpoint proteins to be identified in budding yeast.[83,84] After IR damage, MDC1 becomes hyperphosphorylated in an ATM-, NBS1-, and Chk2-dependent manner,[58,80,81] but MDC1 focus formation is still seen in the absence of ATM.[81] Within 1 min after irradiation, MDC1 forms visible foci that peak in frequency at 30 min.[58,82] MDC1 foci colocalize precisely with γH2AX foci, and γH2AX is needed for MDC1 focus formation.[58,82] H2AX and MDC1 are mutually interdependent for phosphorylation and focus formation, and MDC1 forms complexes with γH2AX.[58] However, there are conflicting data concerning whether MDC1 is required for 53BP1 focus formation.[5882] Suppression of MDC1 by siRNA results in decreased phosphorylation of SMC1^{S996} and Chk1^{S345}.[58] This defect in Chk1 phosphorylation results in defective intra-S phase and G2 checkpoints after IR exposure. Suppression of MDC1 expression by siRNA also causes reduced apoptosis in response to IR damage (because of the loss of the MDC1-Chk2^{P68} interaction[80]), but also decreases colony-forming ability.[82]

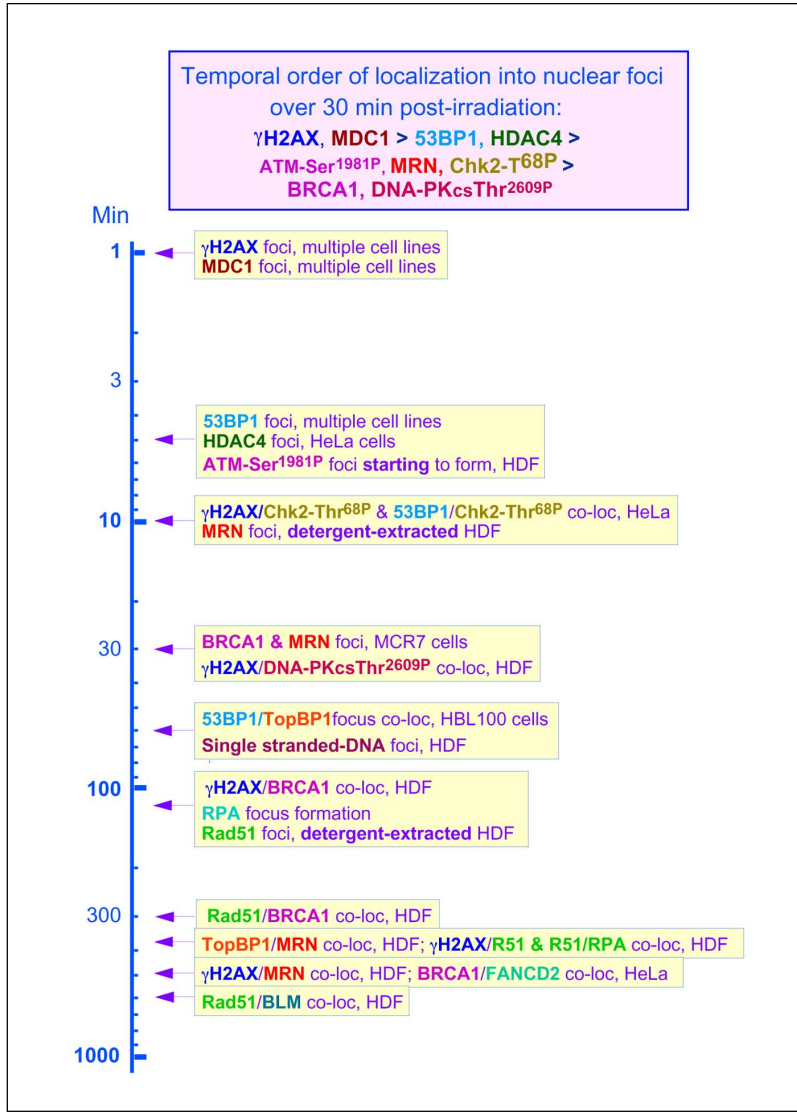

Figure 4. Timeline of recruitment of signaling, repair, and checkpoint proteins to sites of DSBs. Immediately after irradiation the formation of phosphorylated histone H2AX (γH2AX) is detectable and appears to be a/the critical event that modifies chromatin and provides an expansive high affinity site to recruit the repair and checkpoint proteins. The approximate order of appearance of key proteins that have been identified in nuclear foci is indicated. In some instances the position of the arrow may be the earliest time point that was reported, rather than the time of first appearance of the foci. The time of greatest abundance of the foci is often later than when they first appear. The kinetics of appearance of foci containing the MRN (Mre11-Rad50-NBS1) complex or Rad51 is much more rapid in detergent extracted cells.[114] Many studies were done using IR doses in excess of 2 Gy and represent observations on nonsurviving cells. Particularly at later times, foci may represent abortive repair events in dying cells. Most studies have used asynchronous cell populations. Additional work with synchronized cells will provide more precise descriptions of the order of events.

53BP1

Human 53BP1 (1972 a.a. and containing two C-terminal BRCT[85] repeats like MDC1/NFBD1) was identified as a Tp53-interacting protein in a yeast two-hybrid screen[86,87] and was found to have homology with $Rad9^{Sc}$.[88] 53BP1-null (or 53BP1-truncated) mice have a phenotype resembling that of H2AX-deficient mice and that of AT, i.e., growth retardation, immune deficiency, radiation sensitivity, impaired Chk2 phosphorylation/activation (Fig. 2), and cancer proneness.[89,90] Although embryo-derived cultures appear to have intact G1, S, and G2 checkpoints,[89] other studies with different cell types report a requirement for 53BP1 in the S and G2 checkpoints.[57,78]

Cytologically, 53BP1 shows diffuse nuclear immunostaining in undamaged G1 cells but a punctate pattern in S phase,[90] suggesting localization to sites of stalled or broken replication forks. 53BP1 localizes into nuclear foci in most cells as early as five min after IR exposure; doses as low as 0.5 Gy result in formation of these foci.[88] After 1 Gy, the maximal numbers of foci per cell (≈ 20-35) and of foci-positive cells (~92%) are seen between 15 and 120 min.[78,88,91] This number of foci is consistent with the expected yield of DSBs, as discussed above. Significant colocalization of 53BP1 with γH2AX is seen between 10 and 240 min,[78,88,91] and the two proteins show damage-dependent coimmunoprecipitation.[91] As with γH2AX, 53BP1 focus formation is specific for agents that produce DSBs and occurs more rapidly than that of other proteins discussed below (BRCA1, MRN, and Rad51). Phosphorylation and focus formation of 53BP1 are controlled independently.[92] One notable difference between 53BP1 and γH2AX is that only γH2AX forms foci in mitosis,[50,93] where 53BP1 associates with kinetocores.[94]

Recent work shows that 53BP1 has a central role in IR-induced DSB signaling for the S- and G2-phase checkpoints [see editorial in ref. 95]. Upon inhibition of 53BP1 by siRNA, phosphorylation of Tp53 and BRCA1 by ATM is blocked, and the downstream formation of IR-induced BRCA1 foci is largely abolished.[57] Studies with mouse knockout cells show that 53BP1 is required for a normal G2-M checkpoint at low IR doses.[78] In AT cells, 53BP1 focus formation after 1 Gy was diminished at early times (10-20 min post irradiation) in one study,[91] and the hyperphosphorylation that occurs after irradiation was absent or reduced.[91,93] However, in other studies that used 3 or 8 Gy, 53BP1 focus formation appeared to be normal in AT cells,[88,96] perhaps because of the higher doses used. ATM phosphorylates 53BP1 in vitro,[78,91,92] further suggesting that ATM is responsible for directly phosphorylating 53BP1 within γH2AX foci. ATM and 53BP1 also show IR-dependent coimmunoprecipitation.[96] 53BP1 may either help recruit and activate ATM at sites of DSBs, or recruit ATM substrates to these sites.[96] It is noteworthy that 53BP1, as well as Chk2, appears to be present in γH2AX foci before any of the proteins that participate in DSB repair. These results suggest that the initiation of checkpoints precedes the onset of DSB repair, which occurs over a period of several hours, depending on the dose.

HDAC4

Histone deacetylase 4 (HDAC4) is another early participant in IR-induced focus formation, and HDAC4 foci are seen in cells defective in ATM, DNA-PKcs, or NBS1.[97] In contrast, HDAC2 or HDAC6 do not form foci. Interestingly, the stability of 53BP1, as well as its focus formation, shows a dependence on the presence of HDAC4, as shown by siRNA inhibition experiments. Depletion of HDAC4 abrogates the G2 delay and confers sensitization to killing by IR while also reducing cell viability.[97] In this study the authors suggested that the degree of persistence of HDAC4 foci might be a measure of cellular radiosensitivity.

ChK2

Chk2/Cds1 is a key checkpoint kinase (for reviews see refs. 3,98,99), whose role appears to be primarily promoting apoptosis, not cell survival, after IR exposure.[100,101] Chk2-deficient

mice are radioresistant and defective in Tp53-mediated transcriptional changes.[100,101] Thymocytes, splenocytes, skin, and neurons in the developing brain show protection from IR-induced apoptosis. The G1 checkpoint, but not the G2 or S-phase checkpoints, was substantially impaired in *Chk2-/-* embryonic fibroblasts and ES cells.[100,101] IR-induced stabilization of Tp53 in *Chk2-/-* cells is ~60% of that in wild-type cells.[101] Caffeine further reduces Tp53 accumulation, suggesting the presence of another pathway for Tp53 stabilization that is ATM/ATR-dependent, but Chk2-independent. In spite of Tp53's partial stabilization and phosphorylation at Ser^{23} (Ser^{20} in human cells) in the absence of Chk2, Tp53-dependent transcriptional induction of target genes, such as *CDKN1A/p21*, was not observed in *Chk2-/-* cells.

IR treatment results in ATM-dependent activation and phosphorylation of Chk2 at Thr^{68}.[102] The complete activation of Chk2 requires NBS1.[103,104] Immuno-staining using phospho-specific Chk2 antibodies suggests that Chk2-Thr^{68P} localizes into discrete foci within 10 min after irradiation and colocalizes with both γH2AX and 53BP1 (Fig. 4).[57,102] These Chk2-Thr^{68P} foci did not appear in AT cells or in 53BP1-depleted cells, and nonphosphorylated Chk2 molecules remain distributed throughout the nucleus.[57] However, a subsequent study using 53BP1 mouse knockout cells and a different Chk2-Thr^{68P} antibody concluded that 53BP1 is not required for Chk2 activation.[78] These different conclusions might be accounted for by inadequate specificity of the Chk2-Thr^{68P} antibody[102] used in the former studies (see commentary in ref. 95). In summary, phosphorylation and focus formation of Chk2 may be a major determinant in programming cells for elimination by apoptosis through Chk2's site-specific phosphorylation of Tp53.

Chk1

Few Chk1 foci studies have been reported, but Chk1 and BRCA1 foci colocalize in the absence of IR exposure.[105] In comparison to Chk2 described above, after IR damage in chicken DT40 cells Chk1 promotes reproductive survival, apoptosis (in these Tp53-deficient cells), implementation of the G2 checkpoint, and phosphorylation of Cdc2.[106] Notably, *Chk1* null cells completely lose the G2 checkpoint in DT40 cells (see Fig. 2). Inhibition of human Chk1 with siRNA also results in a G2 checkpoint defect, and phosphorylation of Ser^{317} and Ser^{345} appears nonessential for IR-mediated activation of Chk1 and the G2 checkpoint.[107] IR activation of Chk1 depends on BRCA1.[105]

MRN Complex

The analysis of nuclear focus formation by various damage-response proteins suggests that γH2AX formation plays a critical role in recruiting and assembling repair proteins, including the Mre11-Rad50-NBS1 (MRN) complex. This complex is essential for HRR in human cells since hypomorphic mutations confer radiosensitivity.[108-110] MRN localizes to sites of damage within 30 min after irradiation[111] (Fig. 4), and recent genetic evidence suggests that it may be a primary DSB recognition factor.[66] By using a 390-nm laser combined with BrdUrd incorporation and Hoechst dye 33258[112] to produce striped regions of DSBs, Bonner and coworkers have shown in human breast tumor MCF7 cells that γH2AX stripes appear rapidly in all cells, and MRN colocalizes to these stripes within 30 min.[75] Colocalization of NBS1 and γH2AX foci appears to involve a direct interaction between NBS1 and γH2AX (and not H2AX), which is mediated by the FHA/BRCT domain of NBS1.[52]

Initial observations of MRN foci indicated that the kinetics of appearance was too delayed to correspond with productive repair of DSBs.[113] However, the patterns of focus formation of proteins such as Rad50 and Rad51 are strongly influenced by the method of preparing the cells. In earlier experiments using methanol fixation and acetone to permeabilize human diploid fibroblasts, the percentage of nuclei that were positive for Rad50 focus formation after 12 Gy γ-irradiation reached a maximum of ~65% after 8 hr.[113] In comparison, nuclei with Rad51

foci reached a broader maximum between 4-8 hr at ~35%. Subsequent studies in detergent extracted cells, designed to reveal proteins tightly associated with chromatin, found that MRN foci were detectable within 10 min after irradiation and reached a maximum at 2 hr when 70-95% of cells were positive.[114] Since MRN foci are present in AT cells, MRN focus formation does not depend on the phosphorylation of NBS1 by ATM.[61-64] These results suggest that the MRN complex may participate in a very early, ATM-independent step of DSB recognition.[66] Larger aggregates of MRN foci that become apparent by 8 hr in normal cells were not seen in AT cells.[114] These larger, more robust foci may represent sites of slow or abortive repair of complex DSBs. In unirradiated detergent-extracted cells, Mre11 shows a high degree of colocalization with immunostaining of PML bodies, a nuclear depot of many proteins that may help regulate cellular defense against insults such as viruses.[115]

BRCA1 and Rad51

Radiation-induced γH2AX focus formation occurs within several minutes in both MCF7 and IMR90 human cells after exposure to only 0.6 Gy.[50] However, higher radiation doses are usually needed to visualize the foci formed by several key proteins that are recruited to the γH2AX foci. After 12 Gy of IR, the kinetics of BRCA1 focus formation is significantly more rapid than that of MRN focus formation.[75] By 2 hr, 10-15% of IMR90 cells show BRCA1 foci, and these overlap extensively with γH2AX foci.[50] While γH2AX-BRCA1 colocalization is maximal by 2 hr, γH2AX-MRN colocalization increases up to 8 hr. BRCA1 focus formation and hyperphosphorylation after IR is dependent on MDC1.[116] In the absence of detergent extraction, Rad51 foci appear at about the same time as γH2AX-MRN foci, but in different cells.[113] Since MRN acts upstream of Rad51 (see Fig. 5), this observation is paradoxical unless all DSB repair events in a given cell were to occur in a synchronous manner. Rad51 foci become prominent at 6 hr in 20-25% of the cells, when the majority of these foci colocalize with BRCA1 foci. Rad51 foci form relatively slowly, are much less numerous than the estimated numbers of DSBs, and require high doses for their detection. Therefore, their biological significance is still unclear.

In SV40-transformed fibroblasts, the kinetics of focus formation was faster (e.g., γH2AX-BRCA1 colocalization in 45 min) and more cells had Rad51 foci, but the order of foci appearance was the same as for IMR90 cells.[75] The relatively early appearance of γH2AX-BRCA1 foci suggests that BRCA1 might interact directly with DNA breaks.[117]

Pretreatment of MCF7 breast carcinoma cells for 30 min with phosphatidylinositol-3-kinase inhibitor wortmannin, which inhibits ATM and DNA-PKcs, completely blocks γH2AX focus formation after irradiation and prevents the formation of repair-protein foci.[75] Importantly, when added 5 min after irradiation wortmannin has no effect on γH2AX, BRCA1, and Rad51 focus formation. These results emphasize that γH2AX is an early, critical event that initiates DNA repair processes. Consistent with this idea is the finding in mouse *H2AX*$^{\Delta/\Delta}$ knockout B cells or ES cells that BRCA1 and MRN foci cannot form in response to irradiation.[76,77] However, Rad51 foci do form in both these mutant cell types although their intensity seems to be diminished in the mutant ES cells exposed to 20 Gy.[76,77]

DNA-PK

The kinase activity of DNA-PK is required for the efficient repair of DSBs by NHEJ, but the molecular mechanism underlying this activity is not understood.[118,119] Very recently the catalytic subunit of DNA-PK (DNA-PKcs) was found to undergo autophosphorylation in vitro at the highly conserved Thr^{2609} and phosphorylation at this position occurred in vivo in response to IR damage.[120] DNA-PKcs ^{Thr2609} colocalizes within 30 min after IR with both γH2AX and 53BP1. After 10 Gy the phosphorylation of Thr^{2609} is maximal by 30 min and persists for up to 4 hr. The biological significance of this phosphorylation is indicated by the

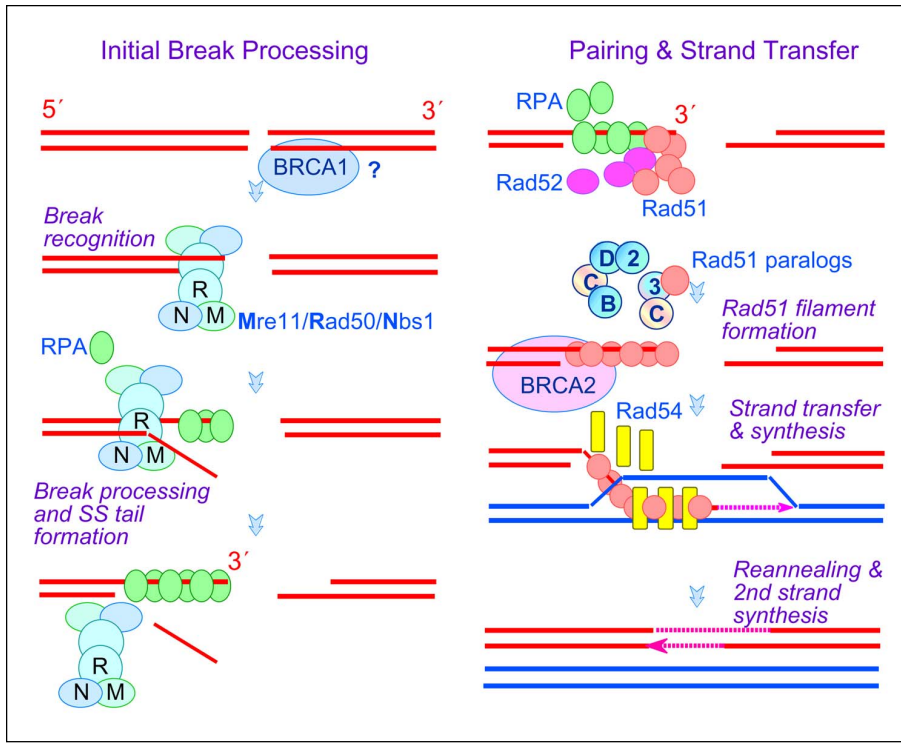

Figure 5. Outline of events in HRR. BRCA1 has an important, undefined role in regulating HRR. The MRN complex likely participates in both the recognition and processing of DNA ends to produce single-stranded tails, which will bind avidly to RPA. Formation of the Rad51 nucleoprotein filament appears to involve the action of BRCA2, the five Rad51 paralogs, and Rad52. Rad52 has functional redundancy with XRCC3,[340] and possibly other Rad51 paralogs.

finding that a Thr2609Ala substitution mutation is associated with defective DSB rejoining and causes increased radiation sensitivity in CHO cells. DNA-PK autophosphorylation at $Thr^{2609}$is reduced in the absence of ATM. Attempts to identify Ku70/86 foci in irradiated cells have generally been unsuccessful in seeing localization of this DNA end-binding complex.[114,121] Immunostaining of Ku70/86, or total DNA-PKcs, in the absence of detergent extraction, does not reveal discrete foci. This specificity suggests that only $DNA\text{-}PKcs^{Thr2609}$ may be needed to recruit Ku70/86 to DSBs, and that a high local concentration of Ku70/86 at a break may be unnecessary because of its abundance in the nucleus.

RPA

The trimeric RPA complex binds single-stranded DNA and is an essential component of both HRR and DNA replication. RPA might provide an appropriate marker for foci in which recombination has been initiated through the processing of DSB ends into structures containing single-stranded DNA coated with RPA. In human cells, interaction between RPA and Rad51 is mediated by the 70-kDa subunit of RPA.[122] RPA foci induced by irradiation are detectable at doses as low as 0.5 Gy and are present at 2 hr (and possibly earlier).[123] In focus-positive cells, the number of RPA foci per nucleus reached a maximum at ~3 hr. It would

be of interest to examine the colocalization of RPA with Rad51 at low doses that allow relatively high cell survival (e.g., 0.5 Gy). This information might allow an estimate of the time required for Rad51 filament formation to occur.

TOPBP1

Human TopBP1 (1522 a.a.) has sequence similarity with other checkpoint proteins (Rad4/Cut5^{Sc}, Dpb11^{Sp}, & Mus101^{Dm}) and contains eight BRCT domains.[124] TopBP1 binds to DNA ends through its BRCT domains,[125] is required for DNA replication, and interacts with Polε.[126] After radiation damage, ATM phosphorylates TopBP1, but TopBP1 focus formation occurs even in AT cells.[127] TopBP1 colocalizes ~50% with 53BP1 within one hr after irradiation and substantially colocalizes with NBS1 and BRCA1 at six hr after irradiation (Fig. 4). Inhibition of DNA synthesis by hydroxyurea results in relocalization of TopBP1 together with BRCA1 to replication forks, suggesting a role for TopBP1 in rescue of stalled forks.[126]

Kinetics of DSB Repair and Contributions of NHEJ versus HRR

It is informative to compare the information on redistribution of repair proteins in (Fig. 4) with the published studies on the kinetics of DSB rejoining using pulsed-field gel electrophoresis. Rejoining experiments are generally conducted at even higher radiation doses than those used in foci studies. Therefore, the kinetics observed may underestimate the rates of DSB repair occurring at physiological levels of damage. Nevertheless, in a variety of vertebrate cell lines, DSB repair measured by electrophoresis or neutral filter-elution exhibits a rapid component with a half-life of ~15 min and a slow component of ≥ 3-5 hr.[14,128,129] SSBs are repaired, more rapidly than DSBs, and with a rapid component that has a half-life of ~ 4 min.[130,131] In a study using premature chromosome condensation to monitor the rate of repair of visible chromosomal breaks after 6 Gy of x-rays, normal fibroblasts eliminated breaks with a half-life of 1.7 hr,[12] which is intermediate between the rapid and slow components determined by electrophoresis.

The analysis of mutant cell lines provides insight into the relative contributions of NHEJ and HRR pathways to the kinetics of DSB repair. In mouse *scid* cells, which have mutant DNA-PKcs, primarily the slow component of the biphasic curve was prolonged after a high IR dose. The final residual level of breaks was the same as in control cells.[132] Similarly, an extensive study of NHEJ and HRR mutants of chicken DT40 cells found that the *ku70* mutant, but not HRR mutants (*rad51*, *rad52*, *rad54*, and *rad51b*), had an increased half-time for DSB rejoining.[14] However, since assays of DNA size typically do not distinguish between correctly and incorrectly rejoined ends, there could well be qualitative differences between the mutants in the two pathways. Correct rejoining events, measured by restriction-fragment analysis in normal human fibroblasts, occur primarily within the first two hours, and misjoining events occur more slowly.[133] Recent work indicating the saturation of HRR at high IR doses (e.g., 20 Gy)[134] suggests that DSB repair studies done at such doses have limited biological relevance. This saturation explains why HRR mutants have not shown defects in physical assays of DSB repair.

HRR As an Error-Free Mechanism of DSB Repair in S and G2 Phases

HRR acts on DSBs that arise at broken replication forks or on DSBs occurring in segments of DNA that have already replicated. In the G1 phase of the cell cycle, homologous chromosomes do not participate in HRR at an appreciable frequency.[135] Since the proteins that mediate HRR in mammalian cells were recently reviewed,[24,25] they will not be discussed in detail. Figure 5 outlines the steps in HRR. BRCA1 and BRCA2 are essential for efficient HRR,[136,137] but the precise biochemical role for BRCA1 remains unclear. The precise functions of the MRN complex are also poorly understood. It may have architectural functions

besides an enzymatic role in end processing as shown in (Fig. 5)[72,138-140] The finding that null mutations in any of the MRN proteins, as well as in Rad51 and BRCA1/2, are incompatible with cell viability (see review in ref. 25) points to their having essential functions that likely coordinate HRR with DNA replication. (This feature of essentialness also imposes limitations in determining the quantitative contribution of HRR to DSB repair.) Hypomorphic mutations in BRCA2 are remarkably similar in phenotype to mutants of each of the five Rad51 paralogs (XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D) (see review in ref. 25). Recent structural studies implicate BRCA2 directly in binding single-stranded DNA and in assembling the Rad51 nucleoprotein filament.[141-143]

Replication Associated DSBs (One-Sided Breaks)

Overview

As cells proliferate, DNA-damaging biochemical reactions produce lesions that interact with the DNA replication machinery. Electron transfer during oxidative phosphorylation produces reactive oxidative species, which generate DNA single-strand breaks and oxidation products; enzymatic activation of procarcinogens generates species that form bulky adducts. Chromosomal discontinuities will arise during S-phase more frequently when replication encounters lesions (SSBs, adducts, oxidized bases, or abasic sites). Thus, a wide range of DNA damages likely give rise to DSBs during DNA replication. To deal with replication-interfering damages, cells possess an impressive array of safeguards that begin with the informational redundancy in the DNA duplex. Multiple, specialized polymerases with lesion-bypass activities help maintain the integrity of the DNA molecule,[144-146] but these error-prone polymerases have a finite coping capacity. Elegant interrelated checkpoint and repair systems are highly integrated with the replication and transcription machinery to prevent broken or rearranged chromosomes from being passed to daughter cells.

A variety of recent studies provide compelling evidence that DSBs normally arise during DNA replication. *First*, null mutations in ATR[147] or the homologous recombination machinery (*mre11*, *nbs1*, *rad50*, and *rad51*mutants) result in cell lethality that is associated with extensive chromosome breakage at metaphase.[148-153] In sperm nuclei replicating in Xenopus egg extracts, DSBs are detected as ends that label with terminal transferase and by the formation of γH2AX.[154] These breaks are only detectable when DNA replicates in the absence of the MRN complex, indicating a vital role for MRN in repairing replication-associated breaks. Since low levels of γH2AX foci are normally present in S phase mammalian cells (less than one visible focus per cell in one study),[134] and γH2AX foci directly correlate with DSBs,[11] S-phase DSBs are likely rapidly repaired. *Second*, an extrapolation of findings in *S. cerevisiae*,[155] based on relative genome size, indicates that ~100 homologous recombination events, which would be initiated by DSBs, might occur in a diploid mammalian cell during each S phase. *Third*, this numerical estimate is similar to that (i.e., >90) derived from the frequency of sister-chromatid exchange and the very low frequency of crossing over during HRR of endonuclease-generated DSBs.[25]

DSBs arising at replication forks are thought to trigger checkpoint signaling by ATR[156,157] and are dealt with by replication-fork restart and recombinational repair mechanisms (see reviews in refs. 158,159). ATR's burden of maintaining chromosome continuity during replication becomes heavier in cells lacking Tp53 (e.g., many kinds of tumor cells) because of a defective G1 checkpoint, which normally allows for the removal of damage before DNA replication.[160] It should be emphasized that DSBs arising at replication forks differ topologically from those produced by IR in that they generally involve the creation of only one double-stranded end, i.e., a one-ended chromosome break.[161,162] Such asymmetric DSBs may be preferentially

recognized by the HRR machinery to accomplish error-free repair, with a lesser role played by the NHEJ machinery during S phase.

This section deals primarily with DSBs that are associated with replication and elicit the "replication checkpoint", triggered by abnormal DNA structures arising as a consequence of a blocked or collapsed (broken) fork. However, another checkpoint pathway in S phase cells has been defined historically and is referred to as the "intra-S" or "S-phase" checkpoint, which occurs when cells are exposed to IR and the DSBs are not fork-associated. When the S-phase checkpoint is activated, the initiation of replication is preferentially inhibited compared with elongation of active replicons. The signal for this inhibition is presumably DSBs although the finding that deficiencies in Msh2 and Mlh1 compromise this checkpoint[163] prompt the question as to whether other DNA lesions may initiate the process. The S phase checkpoint was originally identified by the finding that AT cells displayed "radioresistant DNA synthesis" caused by the lack of inhibition of replicon initiation.[164] Besides ATM,[165] the S-phase checkpoint requires MDC1,[58,80,81] NBS1,[61,70,108] Mre11,[109] Chk1,[107] SMC1,[166] FANCD2,[167] and Msh2/Mlh1.[163] Two complementary pathways for S-phase checkpoint activation, both of which require ATM, have been described. One subpathway operates through the Chk2 kinase and the other through phosphorylated NBS1 and SMC1 (see Fig. 2, lower left).[166,168] The function of phosphorylated SMC1 is not yet known. Thus, the replication and S-phase checkpoints both act to slow the progression of cells through S phase, but act through different pathways (compare Fig. 2 and Fig. 6). The replication checkpoint depends primarily on ATR rather than ATM, as diagrammed in (Fig. 6) Despite these differences, the two pathways exhibit overlap in the activation of their downstream effectors. After IR damage, the G2/M checkpoint requires the cooperation of both ATM and ATR, as revealed from an elegant analysis of single and double mutants.[169]

Replication-Associated Dsbs Arising from a Damaged Template

In the simplest case, a DSB may occur in one daughter chromatid when a replication fork encounters a SSB, which can arise as an intermediate in base excision repair. Under conditions where excess SSBs are present, as during the repair of methylation damage (e.g., MMS exposure), the production of replication-associated DSBs will be exacerbated. When helicase and polymerase activities become uncoupled at the replication fork[170-172] and generate extended regions of single-stranded DNA (e.g., 1 kb), the likelihood of disrupting chromatid continuity may increase because of increased exposure of SSBs. Moreover, new SSBs might arise from the nicking activity of nucleases acting on single-stranded DNA.

When replication forks encounter polymerase-blocking lesions such as bulky adducts, DSBs can arise by several processes. A blocking lesion in the leading strand can result in the generation of extensive downstream ssDNA in that strand (Fig. 7A). These single-stranded gaps have been documented experimentally during SV40 replication that initiates upstream of a pyrimidine-dimer[173,174] and are favored under conditions when damage bypass of specific lesions is inhibited.[144,175] Under these conditions, ssDNA is generated as the replication fork proceeds while chain extension on the leading strand is restricted. The resultant DNA structures can become destabilized and cause the marked increase in DSBs seen after UV irradiation of both yeast[176] and hamster cells[177] containing photolytic lesions induced by UVA in BrdUrd-substituted DNA in the presence of a photosensitizing dye. This phenomenon is greatly exaggerated in bypass-deficient xeroderma pigmentosum variant (XP-V) cells, which are defective in the bypass polymerase Y/η (discussed further below).[178,179] Regions of ssDNA are also candidates for the formation of secondary structures such as hairpins and cruciform structures that can be recognized and cleaved by the MRN complex or other enzymes.[180-183]

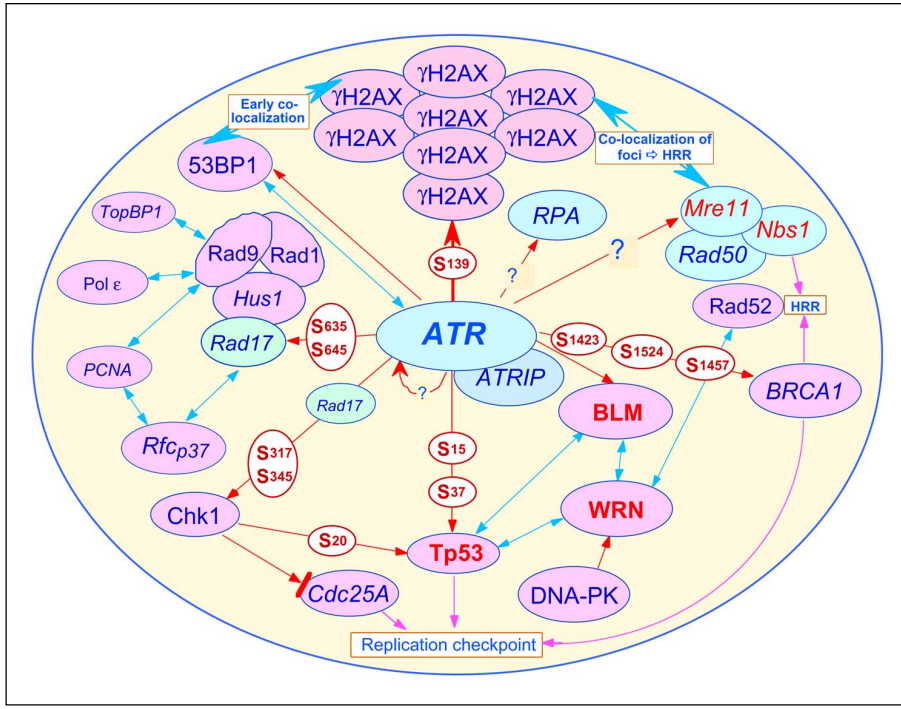

Figure 6. ATR-mediated phosphorylation events and interactions that implement signaling, repair, and checkpoint functions in response to replication-associated DSBs. ATR has diverse substrates that overlap with those of ATM. Proteins with names in red are involved in human genetic disorders and proteins in italics are required for viability of dividing cells. Site-specific ATR mediated phosphorylation events have been determined for H2AX,[251] ATRIP (ATR interacting protein),[210] BRCA1,[221,222] Tp53,[218,219,341] Chk1,[214,246,342] and Rad17.[246,343-345] ATR additionally phosphorylates BLM,[292] and possibly RPA in response to replication-associated DSBs (K. Cimprich, personal comm.). WRN is phosphorylated by DNA-PK, which inhibits its activity.[346] The interactions marked by blue arrows have been reported for ATR-53BP1,[90] BLM-Tp53,[302,347,348] BLM-WRN,[349] WRN-Tp53,[302,350] WRN-Rad52,[351] Rad9-TopBP1,[126] Rad9-Polε,[126] and (Rad9-PCNA, PCNA-RFCp37, RFCp37-Rad17).[352] Kinase Chk1 contributes to S and G2 checkpoints by phosphorylating and inactivating a key checkpoint phosphatase, Cdc25A.[353-355]

Under certain circumstances stalled replication forks can regress or reverse to generate a 4-stranded structure (often called a "chickenfoot"[184]) that can be considered a topologically masked DSB (Fig. 7E). Recent electron micrographs reveal the presence of such structures in yeast *rad53* kinase checkpoint mutants.[185] However, in mammalian cells early studies cast doubt on the existence of these structures. The appearance of doubly-dense DNA from cells pulse labeled with BrdUrd was found to decrease dramatically upon crosslinking with psoralen, suggesting that branch migration occurred as an artifact of DNA isolation.[186,187] Regressed forks are structurally similar to, but topologically distinct from, Holliday junctions (HJs), a common intermediate produced during the repair of DSBs by HR (Fig. 7C). These 4-stranded structures generated by fork regression can be unwound by the structure specific helicases WRN and BLM[188-190] and cut by resolvases.[191,192] Thus, branched intermediate structures that arise at sites of stalled replication can be converted to DSBs in a manner dependent upon their structural and topological context, as further addressed below.

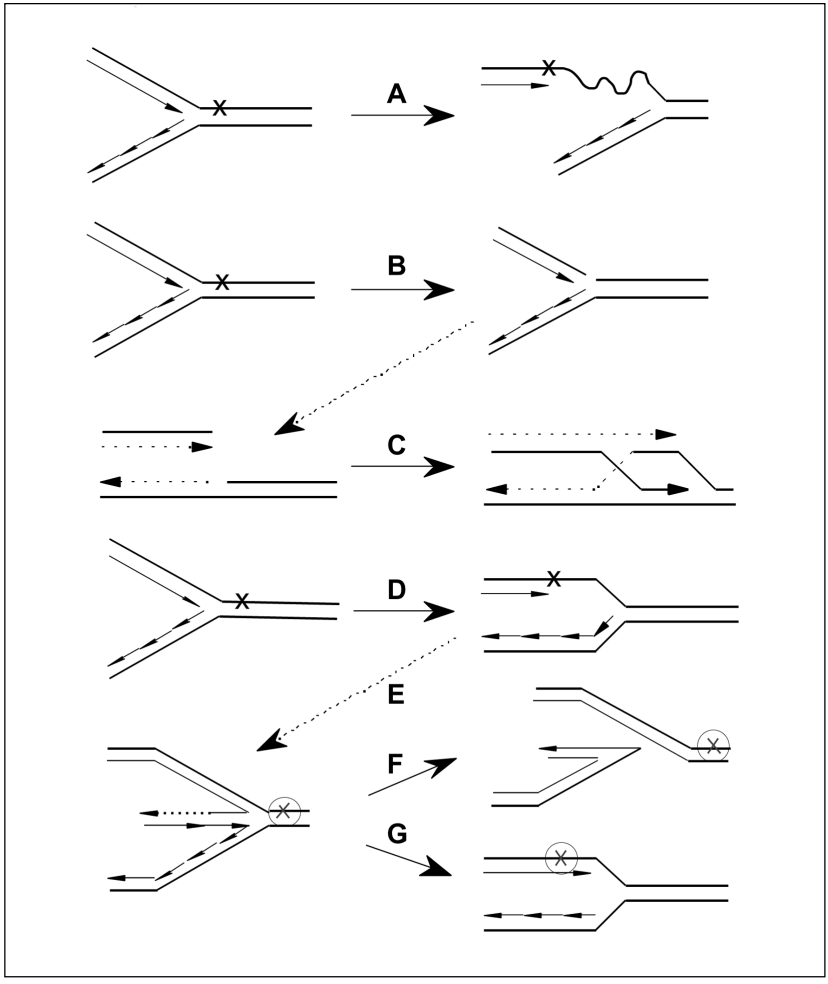

Figure 7. Formation and repair of DSB arising when replication forks encounter damage. (A) Arrest of the replication fork. (B) Fork collapse and DSB generation upon the encounter of the replication fork with a preexisting nick or gap. (C) Formation of a Holliday junction (HJ) at the site of a broken fork. (D) Replication fork encounters a leading strand lesion, designated "X", resulting in the generation of single-stranded DNA. (E) Fork regression (reversal) by branch migration generates a four-stranded chicken-foot structure; extension of the leading strand may occur in the sequence corresponding to that of the site of the lesion. (F) Resolution of the HJ by resolvase, resulting in an incompletely replicated chromosome. (G) Branch migration resets the replication fork with the lesion either present or absent.

Replication-Associated DSBs Arising from Inhibition of Replication

As in *E. coli* and yeast,[193-195] stalled replication forks caused by DNA synthesis inhibitors in mammalian cells are an efficient source of DSBs, cytotoxicity, and potentially deleterious recombination.[196] Inhibition of DNA replication by hydroxyurea and aphidicolin produces chromatid discontinuities at replication forks.[197,198] DSB-promoted intrachromosomal recombination of a partially duplicated *HPRT* gene in hamster cells was most strongly induced by inhibitors of DNA synthesis (hydroxyurea, methotrexate, aphidicolin, cytosine arabinoside),

followed by the topoisomerase inhibitors (camptothecin, etoposide), then bifunctional alkylating agents (cisplatin, mitomycin C), and lastly spindle poisons (vincristine).[199] Camptothecin-induced DSBs associated with replication forks in synchronized cells were detectable by PFGE and were repaired primarily by HRR, not NHEJ.[199]

Although both HRR and NHEJ mediate the resolution of stalled forks during replication arrest, analysis of mutant phenotypes suggests the two pathways play different roles, which likely depend on the nature of fork damage.[197] There is some evidence that blocked forks that have not collapsed promote HRR,[197] but this issue needs further study. Related studies found a differential involvement of NHEJ and HRR in the resolution of DSBs accumulated during extended (6-24 hr) arrest induced by replication inhibitors.[200] The data suggest that NHEJ precedes HRR in the resolution of arrest-induced DSBs, and that HRR becomes more important with extended replication arrest induced by inhibitors.[200,201]

Recent studies in permeabilized cells treated with cisplatin provide insights into how chromosomal fork progression is controlled after DNA damage in vertebrates.[202] Cisplatin DNA adducts *actively* slow fork progression by a process that requires XRCC3, a Rad51 paralog that participates in homologous recombination.[203] The addition of purified human RAD51C-XRCC3 protein complex restores slowing of fork progression in permeabilized XRCC3$^{-/-}$ cells.[202] Moreover, this requirement for XRCC3 is alleviated by adding human Rad51 protein, but not by Rad52 or the recombination complex Rad51B-Rad51C-Rad51D-XRCC2. These data demonstrate that XRCC3 and Rad51 cooperatively modulate the progression of replication forks on damaged vertebrate chromosomes.

Recent results suggest that the mode of DSB repair at arrested replication forks depends on the functional status of Tp53 (see also discussion on WRN and BLM below). The induction of HRR during the inhibition of replication is stimulated by mutant Tp53,[204] and stalled replication forks produced by hydroxyurea and aphidicolin stimulate the accumulation of nuclear Tp53 that is impaired and altered in its transcriptional activity compared with Tp53 induced by IR.[205] In XP-V cells following UV-induced replication arrest, inactivation of Tp53 by SV40 or HPV16 transformation stimulates DSB-dependent modification and relocalization of the MRN complex.[178,179,206] High wortmannin concentrations, which inhibit PI3KKs (phosphatidylinositol-3-kinase related kinases), lead to a marked increase in the number of transformed XP-V cells exhibiting MRN foci following UV irradiation.[207]

Recognition and Signaling of Stalled and Collapsed Forks

ATR-Mediated Signaling

Creation of DSBs at sites of arrested forks can occur when enzymatic processing of the blocked structures leads to breakage of parental DNA strands, causing what is termed fork collapse (Fig. 7B). The details of how mammalian cells recognize and signal the presence of specific replication anomalies are beginning to be understood. Although the abnormal DNA structures that are initially recognized as critical replication fork "damage" are not yet well defined, the ATR kinase is a key early component in the signaling process.[3,38,169] (The homologs of ATR in yeasts are Rad3^{Sp} and Mec1^{Sc}.) It is not entirely clear whether replication-associated γH2AX formation *always* requires collapse of a replication fork. However, the fact that a 3 hr aphidicolin treatment did not produce γH2AX foci strongly suggests that the signal arises from DSBs rather than stalled forks.[53] (Much longer aphidicolin exposure causes γH2AX focus formation).[134] In case of treatment with camptothecin, which binds Top1, DSBs associated with the cleavage complex at replication forks result in ATR-dependent γH2AX focus formation; DNA-PK and ATM also contributes to this phosphorylation.[53] Examples of γH2AX foci seen in both CHO and human cells are given in (Fig. 3), panels B-D.

Even though ATR and ATM are functionally related, they differ very significantly. In contrast to ATM, which responds primarily to DSBs occurring outside the context of replication arrest, the ATR kinase prevents the accumulation of DSBs during replication arrest, as from aphidicolin treatment (Fig. 5).[3,169,208] Unlike ATM, deletion of ATR function leads to early mouse embryonic and cellular lethality.[147,209,210] ATR, but not ATM, plays a critical role in preventing chromosomal gaps and breaks at fragile sites (as well as at random sites), which are greatly enhanced by inhibiting replication with aphidicolin.[198]

The response of ATR to replication stress has provided many of the insights into its essential cellular functions as a regulator of replication arrest. Overexpression of kinase-inactive ATR mutants in human fibroblasts produces dominant negative phenotypes that show abnormalities in cell cycle progression, reduced phosphorylation of signaling and repair proteins, and elevated sensitivity to killing by DNA damaging agents (IR, UV, MMS, the topoisomerase I inhibitor topotecan, & the topoisomerase II inhibitor etoposide) and replication blocks (e.g., hydroxyurea). [42,211,212] In Xenopus egg extracts and mammalian cells, ATR is required both to phosphorylate Chk1 in response to replication arrest and to properly activate the replication checkpoint.[213-215] In the Xenopus extracts, replication forks were shown to be an obligate intermediate for the activation of this checkpoint.[216,217] In addition to phosphorylating Chk1, ATR plays a role in regulating the replication checkpoint through phosphorylation of Ser15 on Tp53, which is also a target of the Chk1 kinase (Fig. 5).[3,214,215,218-220] ATR and p53 can function independently, but loss of both can cause synergistic disruption of the replication checkpoint.[219] In response to replication stress induced by UV-dimer photoproducts or replication inhibitors, ATR redistributes to form nuclear foci, presumably at stalled or broken replication forks.[90,221,222] ATR interacts with 53BP1, phosphorylates it in vitro, and colocalizes with it after replication inhibition.[90] ATR also phosphorylates BRCA1 with an overlapping spectrum of sites compared with ATM, and forms foci that partially colocalize with BRCA1 foci.[221,222]

ATR appears to act, not only in response to drug-imposed blocks to replication (e.g., aphidicolin, hydroxyurea), but also as an intrinsic replication fork checkpoint initiator that is normally active throughout S phase.[222] Experiments using Xenopus extracts have shown that this checkpoint function depends on the replication-dependent chromatin-binding properties of ATR.[223] After the initiation of replication, ATR binds to chromatin, where it can phosphorylate a range of downstream effectors and then dissociate upon the completion of replication.[216,223] Nuclei in Xenopus extracts treated with replication inhibitors accumulate ssDNA, RPA, and γH2AX foci, consistent with the production of replication-associated DSBs.[224] In the absence of inhibitors, replication-associated RPA coating ssDNA appears to facilitate the binding to chromatin of both ATR and the checkpoint protein Hus1 (hydroxyurea sensitive); the recruitment of Polα is also required for chromatin association of Hus1.[172] Recruitment of the Hus1 protein complex to chromatin is independent of ATR binding, and checkpoint activation requires RNA synthesis by Polα.[172,225] Since both ATR and Hus1 are required for the phosphorylation/activation of Chk1,[172] their coincident binding to chromatin is likely critical in activating ATR either by interaction with Hus1 or by recruiting Chk1 to the chromatin.

Improper execution of the replication checkpoint may account for the chromosomal fragmentation and early embryonic lethality observed in ATR null mouse embryos[147,209] and in Chk1 null mice.[226] Somewhat surprisingly, incomplete DNA replication in mouse cells after aphidicolin treatment can prevent M-phase entry independently of ATR and inhibitory phosphorylation of Cdc2.[169] However, when the replication inhibitor is removed, ATR knockout cells proceed to mitosis with extensive chromosome breaks, indicating that ATR provides a key genome maintenance function in S phase.[169]

ATR may act directly as a DNA damage sensor during replication arrest. ATR preferentially binds to UV-damaged DNA in vitro.[227] This binding depends on UV fluence and

full-length ATR, and results in a stimulation of its kinase activity. The binding partner of ATR, ATRIP (hRad26), was recently identified as the human homolog of $Ddc2^{Sc}$ (also called Lcd1 or Pie1) and $Rad26^{Sp}$.[210] Observations that ATRIP associates with ATR, is a substrate of ATR, and is a phosphoprotein in vivo,[210] are compatible with similar interactions found for the yeast homologs Mec1-Ddc2 in *S. cerevisiae* and Rad3-Rad26 in *S. pombe*.[228-231] DNA damage and replication inhibition cause ATRIP to colocalize with ATR.[210] Deletion of ATRIP reduces the level of the ATR protein and generates checkpoint defects similar to those of an ATR deletion,[210] which suggests that ATRIP and ATR are mutually dependent in signaling and checkpoint pathways. It remains unclear whether ATRIP recruits and/or stimulates the association of ATR to replication-associated DSBs.

The Rad17 and Rad9 Complexes

The ATR/ATRIP complex interacts with other early damage sensor elements during checkpoint activation. Specifically, the mammalian checkpoint proteins Rad17 (RFC1 homolog; RFC = replication factor C)[232-234] and the Rad9-Rad1-Hus1 (9-1-1) complex[235-237] are named after their counterparts in *S. pombe*.[4] (The corresponding proteins in *S. cerevisiae* are Rad24, Rad17, Ddc1, and Mec3, respectively.) While direct evidence is lacking that the Rad17-RFC heteropentameric complex and the 9-1-1 complex actually recognize replication-associated DSBs or other abnormal DNA structures at arrested forks, their interactions with chromatin and ATR appear to be critical in the early recognition and signaling of replication arrest. The Rad17-RFC and 9-1-1 complexes show sequence and structural similarity to the clamp loader (RFC) and sliding clamp (PCNA) complexes required for replication.[216,232,238-243] The biological importance of Rad9 in IR sensitivity is illustrated by the strong phenotype of *rad9* knockout mouse ES cells, which display increased spontaneous chromosomal aberrations, IR and UV sensitivity to killing (3-fold), and a partially defective G2 checkpoint.[250] Homozygous mutant embryos die between E9.5 and E12.5.

It was recently inferred that the checkpoint role of the 9-1-1 complex is not restricted to S phase and replication blockage. DNA-damage-induced binding of Rad9 to chromatin occurred in noncycling cells after exposure to IR or a bulky-adduct mutagen.[244] However, the dose of IR used (50 Gy) may produce interactions that are irrelevant to normal physiological responses. In cycling cell populations, the IR- and hydroxyurea-induced binding of Rad9 (and Rad1) to chromatin occurs independently of the ATM phosphorylation of Rad9 at Ser^{272} (Fig. 2) and PIKK activities. Phosphorylation is also not required for Rad9's interaction with Rad1, Hus1, and Rad17.[244]

The critical role of Rad17 in preventing accumulation of DNA DSBs during replication is revealed by the properties of a *Rad17flox/-* conditional mutant in human HCT116 cells.[245] Loss of Rad17 causes rapid accumulation of chromosomal breaks and rearrangements as well as endoreduplication. However, the chromosomal breakage in *rad17* null cells was less severe than that of *atr* null cells examined in parallel.[245] Rad17 null cells have defective Chk1 phosphorylation after UV damage, normal phosphorylation of ATM targets including Chk2 after IR damage, and a partially defective G2 checkpoint after IR damage.

Rad17 is constitutively bound to chromatin in human cells (although this was not seen in Xenopus egg extracts without DNA damage[217]) and is phosphorylated by ATR on chromatin after treatment with UV radiation, γ-rays, and hydroxyurea.[246] Rad17, but not its phosphorylation by ATR, is required for loading the 9-1-1 complex onto chromatin (Fig. 6) in a manner analogous to PCNA loading by RFC. However, phosphorylation of Rad17 is required for the downstream phosphorylation/activation of Chk1. Both ATM and ATR are required for Rad17 phosphorylation in response to IR at early times, but UV-induced Rad17 phosphorylation appears to be specifically produced by ATR.[246] Hydroxyurea-induced Rad17 phosphorylation is partially dependent on ATR but independent of ATM in the presence of ATR.[246] Similar to

Xenopus mentioned above, the 9-1-1 and ATR-ATRIP complexes in human cells can be recruited to chromatin independently (in response to UV damage). Both complexes are present at sites of UV damage as indicated by the partial colocalization of ATR foci with Rad17-Ser^{635} foci. Thus, Rad17's interactions with the 9-1-1 complex may help determine the selection of substrates available to ATR.[246] In *S. pombe* recent evidence indicates that the DinB damage-response polymerase physically interacts with the 9-1-1 complex and requires Rad17 to associate with chromatin, suggesting that the checkpoint response includes translesion synthesis.[247] One model of checkpoint activation is that the loading and interaction of the ATR-ATRIP and 9-1-1 complexes, mediated by Rad17, creates a higher-order chromatin structure to facilitate signaling and phosphorylation of Chk1.[246] Such a chromatin change might be analogous to that which appears to be mediated by ATM already discussed.[48]

This idea is compatible with the finding that Hus1 acts upstream of Chk1 and is required for its optimal phosphorylation in mammalian cells.[248] Hus1 is not required for Tp53 accumulation and activation or for Chk2 phosphorylation. Disrupted signaling during replication stress likely underlies the embryonic lethality in Hus1-deficient mice.[249] Hus1-deficient embryonic fibroblasts have been rescued for in vitro viability by simultaneous disruption of CDKN1A/p21,[249] and these cells exhibit chromosomal instability, heightened sensitivity to replication blocks, and altered cell cycle responses. They display high sensitivity to UV radiation and hydroxyurea but only slight IR sensitivity,[248,249] which is consistent with the idea that the 9-1-1 complex functions in the response to replication-associated damage. The lesser IR sensitivity of *hus1* mutant cells[248] compared with *rad9* cells[250] suggests that these two complex members have overlapping but not identical functions.

Factors Promoting the Repair of Replication Associated DSBs

As already discussed, overt DSB production leads to the production of γH2AX, a large-scale chromatin modification that can be visualized as nuclear foci. Exposure of mammalian cells to UV radiation or hydroxyurea leads to ATR-dependent phosphorylation and γH2AX focus formation.[251] Colocalization of these γH2AX foci with PCNA in S-phase synchronized cultures suggests that these foci are associated with sites of replication fork arrest.[251] The idea that these γH2AX foci reflect DSBs is supported by the finding that both UV radiation and hydroxyurea efficiently induce sister-chromatid exchange,[252] a manifestation of DSB repair by homologous recombination involving crossing-over.[253] Replication-dependent formation of γH2AX foci is also seen in both human cells [53] and nuclei incubated in Xenopus egg extracts [224] after treatment with the topoisomerase I poison camptothecin. In the Xenopus system, the induction of γH2AX foci is inhibited by geminin, a replication licensing inhibitor.

Additional evidence supporting the importance of γH2AX after S-phase DSB formation comes from studies utilizing replication-defective xeroderma pigmentosum variant (XP-V) cells. Exposure of XP-V cells to UV radiation leads to a fluence-dependent increase in the fraction of cells showing γH2AX foci, which are illustrated in (Fig. 3) These foci are only observed in S-phase cells, and they coincide with PCNA and MRN foci, further supporting the concept that the chromatin modification associated with H2AX phosphorylation recruits HRR proteins that facilitate repair between sister chromatids.[178,179] Moreover, cells derived from H2AX null mice show a decrease in the portion of proliferating cells and an increased level of S-phase-derived chromatid aberrations.[76] These observations, coupled with data showing that *H2AX*$^{\Delta/\Delta}$ ES cells exhibit reduced HRR[76] but normal NHEJ,[77] suggest that chromatin modification involving H2AX is critical in protecting cells against replication-associated genomic instability associated with aberrant recombination.

The Mre11 complex plays an important role in repairing replication associated DSBs. MRN exhibits extensive colocalization with PCNA throughout S phase, and replication fork stalling imposed by hydroxyurea enhances the chromatin association of MRN.[114,254] As in

XP-V cells discussed above, these results further suggest that MRN loads onto chromatin at blocked or collapsed replication forks. This idea is supported by the observation that MRN preferentially localizes to single-stranded DNA arising in hydroxyurea-treated cells.[254] In camptothecin treated cells, MRN focus formation requires γH2AX formation, and H2AX null mouse cells are hypersensitive to killing by camptothecin.[53] The finding that aphidicolin blocks camptothecin-induced γH2AX focus formation shows that replication produces these DSBs.[53]

TopBP1 appears to be another key protein involved in preventing replication-associated chromosomal rearrangements. TopBP1contains eight BRCT domains, which mediate multiple interactions, and has sequence homologs in yeast[255,256] and flies.[257] These homologs play important roles in DNA replication, repair and checkpoints in lower organisms.[257-261] In addition to facilitating normal replication through its interaction with Pol-ε, TopBP1 responds to the inhibition of DNA synthesis by localizing with other repair proteins (BRCA1, PCNA) during S-phase, suggesting a possible role in rescuing stalled forks.[126,127] TopBP1 localized at sites of replication arrest may act to relieve torsional stress developed during the generation of anomalous DNA structures. Replication stress also elicits focus formation for 53BP1,[91] a protein discussed earlier in the context of IR damage.

Processing Abnormal Replication Intermediates and Associated DSBs

Helicases and topoisomerases are specialized enzymes that modify the three dimensional structure of DNA. Helicases increase accessibility of the replication and repair machinery to DNA by locally unwinding the duplex. Topoisomerases modulate the torsional strain of the DNA helix by catalyzing the interconversion of topological isomers. Known interactions between these two classes of proteins suggest a need to colocalize their activities to resolve abnormal DNA structures that can arise at stalled forks.

The RecQ family of helicases is critical to the maintenance of genomic integrity. These helicases (see reviews in refs. 190,262,263), named after the *E. coli recQ* gene product, include yeast $Sgs1^{Sc}$[264,265] and $Rqh1^{Sp}$,[266,267] and five members in humans: BLM,[268] WRN,[269] RecQ1/RecQL,[270,271] RecQ4,[272] and RecQ5.[272-274] Deficiencies in the BLM, WRN, and RecQ4 helicases cause Bloom and Werner syndromes,[268,269] and some cases of Rothmund-Thomson syndrome.[275-277,279] These rare genetic diseases manifest distinct yet overlapping clinical phenotypes of immunodeficiency, premature aging, chromosomal instability, and predisposition to cancer (see reviews in refs. 25,189,190,280-282).

The mutations in BLM and WRN helicases are associated with replication defects, including impaired progression of replication forks, an accumulation of abnormal replication intermediates,[283-285] and aberrant homologous recombination.[286,287] BLM- and WRN-defective cells display an abnormally high percentage of deletion mutations at specific loci.[288,289] BLM and WRN helicases can suppress the increased homologous and illegitimate recombination in the *S. cerevisiae sgs1* mutant.[290] Elevated sensitivity to replication-blocking inhibitors is seen in Bloom syndrome (BS) cell lines[291-294] and in WRN-deficient cells.[295-299] Conflicting results are reported concerning altered sensitivity of BLM-deficient cells to inhibition by hydroxyurea.[291,292,294] WRN mutant cell lines consistently show hypersensitivity to camptothecin and defective responses to hydroxyurea.[295-299]

It has been proposed that DSBs formed during replication arrest can lead to the formation of HJs (see Fig. 7C), substrates recognized by the BLM and WRN helicases.[188,300,301] The ability of these helicases to unwind HJs is dependent on Tp53, which binds to the enzymes and attenuates their branch migration activity, and possibly their anti-recombinase functions.[302] Tp53 and BLM functionally interact during resolution of stalled DNA replication forks.[278] The evidence supports a model in which the disruption of abnormal structures by the BLM and WRN helicases prevents aberrant recombination events that would result in chromosomal rearrangement (see Fig. 7G).[188,287,300,301,303] The interaction between BLM and topoisomerase

IIIα (TopIII) in human cells, which is highly conserved across eukaryotic species,[264,304-306] may also promote unlinking of the parental duplex by TopIII at sites of paused or convergent replication forks.

Once formed, HJs may elude the activity of helicases and be cleaved by endonucleolytic HJ resolvasesto generate DSBs. Much of our understanding of these reactions comes from the genetic studies in bacterial systems characterizing the branch migration complex RuvA/RuvB, and the RuvC and RusA resolvases (see ref. 192 for a brief review). Activities from mammalian cell extracts that resemble those expected for a true HJ resolvase have been identified,[307,308] but it is unclear whether these activities correspond to the newly described mus81 proteins in yeast and humans.[191,309-311] Nonetheless, the Mus81 homologs appear to specifically cleave replication intermediates that possess branched structures, and Mus81 (i.e., the Mus81-Eme1 heterodimeric endonuclease) is particularly important in *S. pombe* in processing stalled or collapsed forks in a RecQ helicase-deficient background.[311]

Recent work shows an important link between the BLM helicase and the MRN complex during replication arrest. In response to hydroxyurea, the formation of MRN foci at sites of stalled forks was sharply reduced in Bloom syndrome cells.[292] However, in nonBS cells, ATR-dependent phosphorylation of BLM was not required for subnuclear relocalization of MRN.[292] Function(s) of the MRN complex are suggested by its unique architecture.[72,139,140] The Rad50 zinc-hook motif, through the interaction of two Rad50 "tails", provides a means of tethering sister chromatids for recombinational repair, thereby limiting the undesirable dissociation of broken DNA ends. After fork arrest, the MRN complex may assist in repairing DSBs both by promoting inter-sister connectivity lost during fork regression and by helping to restore chromatid continuity during fork breakdown or HJ resolution. Notably, MRN foci that appear in S phase detergent-extracted cells colocalize with PCNA foci during DNA replication and appear normal in $H2AX^{\Delta/\Delta}$ mutant cells.[76]

Outlook

We discussed what is known about the events of DSB formation, recognition, and signaling, which facilitate the recruitment of checkpoint and repair proteins to these lesions. Homologous recombination may well have arisen early in life forms as a mechanism for ensuring chromosome continuity in the face of DSBs that arise normally during replication. The large genomes of higher eukaryotes must achieve remarkable accuracy in rapidly detecting each DSB and announcing its presence through signal amplification and transduction in order to keep the genome intact in each daughter cell at mitosis. The discovery of γH2AX foci as a likely bona fide marker and sentinel for DSBs represents a major advance.[11,49,50] It appears likely that γH2AX foci will provide a reliable method of quantifying DSBs and their repair.[11] Just how this modification is triggered through ATM's chromatin sensing capacity remains to be determined. Additional candidates for the initial sensors, which help mediate γH2AX formation, are under intense investigation.

Recent research brings the promise of providing the cytological tools needed to accurately quantify the levels of DSBs in fixed cells, and perhaps eventually in living cells.[73] Measuring the very early colocalization of 53BP1 and MDC1 with γH2AX may add robustness to experiments designed to accurately measure levels of DSBs at low IR doses. Better quantitative methods of image analysis are greatly needed to remove the subjectivity and labor inherent in the visual scoring of cytological foci. Since rapid progress has been made in recent years in this field, it seems very likely that we will soon have a detailed understanding of how cells cope so efficiently with DSBs.

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